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# Differences in Destruction of Cysts of Pathogenic and Nonpathogenic *Naegleria* and *Acanthamoeba* by Chlorine

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The destructive action of chlorine on the pathogenic *Naegleria fowleri* and *Acanthamoeba culbertsoni*, the nonpathogenic *N. gruberi*, and an avirulent *Acanthamoeba* isolate was investigated. *N. fowleri* is somewhat more sensitive to chlorine than *N. gruberi*, whereas the two *Acanthamoeba* strains are very resistant. This study yields information needed for the destruction of amoebic cysts in drinking water and swimming pools. It also gives some explanation for the occurence of *Acanthamoeba* strains in these waters.

Much concern has been given recently to the occurrence of pathogenic free-living amoebae in tap water and swimming pools, but little is known about their ecology and resistance to water disinfectants. Since more cases of primary amoebic meningoencephalitis are recognized throughout the world, attention is drawn to *Naegleria* and *Acanthamoeba*, the causative agents of this disease. The cysticidal action of chlorine, the most common disinfectant for treating drinking water and swimming pools, was investigated in this study. The purpose was to ascertain the difference in chlorine sensitivity between pathogenic and nonpathogenic *Naegleria* and *Acanthamoeba* to understand their occurrence in treated waters and to be able to prevent amoebic contamination of drinking water and swimming pools.

Experimental data on the destruction of these amoebae have been very scanty. Duma et al. (8) reported that *Naegleria fowleri* is more resistant to chlorine than *Entamoeba histolytica* and mentioned 4 μg/ml as an effective concentration, without giving contact time and pH. Anderson and Jamieson (1) failed to eradicate *Naegleria* from a swimming pool by superchlorination to 10 μg/ml. Derreumaux et al. (7) concluded from their laboratory experiments that the growth of *Acanthamoeba* and *Naegleria* is prevented in swimming pools by concentrations of 0.5 to 1.0 μg of active chlorine per ml.

## MATERIALS AND METHODS

**Amoeba strains.** *N. fowleri* HB-1 was isolated by Culbertson et al. (4) in the United States from a human fatal case of primary amoebic meningoencephalitis, described by Butt et al. (3), in 1966 (Fig. 1A). *Naegleria gruberi* 1518/1e was isolated by F. C. Page (10) in the United States in 1964 from a millpond (Fig. 1B). *Acanthamoeba culbertsoni* A-1 was isolated by C. G. Culbertson (5) in the United States in 1958 from a tissue culture. This strain is pathogenic for mice (Fig. 1C). *Acanthamoeba* sp. 4A was isolated by us in Belgium in 1974 from tapwater. This strain is tentatively identified as *A. polyphaga* and showed to be avirulent to mice (Fig. 1D).

**Culture methods.** For cyst formation all strains were grown in 1-liter Roux bottles containing 70 ml of liquid MYAS medium in association with living *Escherichia coli* incubated at 37 C (6). The cysts used in the experiments were harvested from 1- to 2-week-old cultures. To determine cyst survival, nonnutrient agar spread with living *E. coli* (NNE) was used as culture medium.

**Experimental procedure.** The cysts were scraped from the vessel wall and centrifuged for 5 min at 750 × *g*. The sediment was washed three times with sterile distilled water. In each experiment $2.5 \times 10^5$ cysts were added to 250 ml of chlorine solution, giving a cyst density of $10^3$/ml. Chlorine solutions were prepared by dilution of commercial sodium hypochlorite solution ("Eau de Javel"). The chlorine solutions (250 ml) were kept in 300-ml Erlenmeyer flasks. The solutions were buffered at pH 7.3 to 7.4 by phosphate buffer. These test solutions were kept in constant motion by means of a magnetic stirrer at a temperature of 25 C.

Free chlorine concentrations were determined by the Palin method (11) with *N,N*-diethyl-*p*-phenylenediamine (BDH Lovibund 1000 comparator). Determination of combined and free chlorine gave the same results; therefore it was concluded that only free chlorine was available in the test solutions. After adding the cyst suspension, two 10-ml samples were taken at given intervals (1, 5, and 15 min; 1, 3, and 24 h). One sample was used for direct determination of free chlorine concentration by the Palin method (11). A crystal of sodium thiosulfate was added for dechlorination to the second sample. After dechlorination this 10-ml sample was filtered through a 0.45-μm membrane filter (Swinnex 25; Millipore Corp.). This concentration method would theoretically give $10^4$ cysts when no deaths had oc-

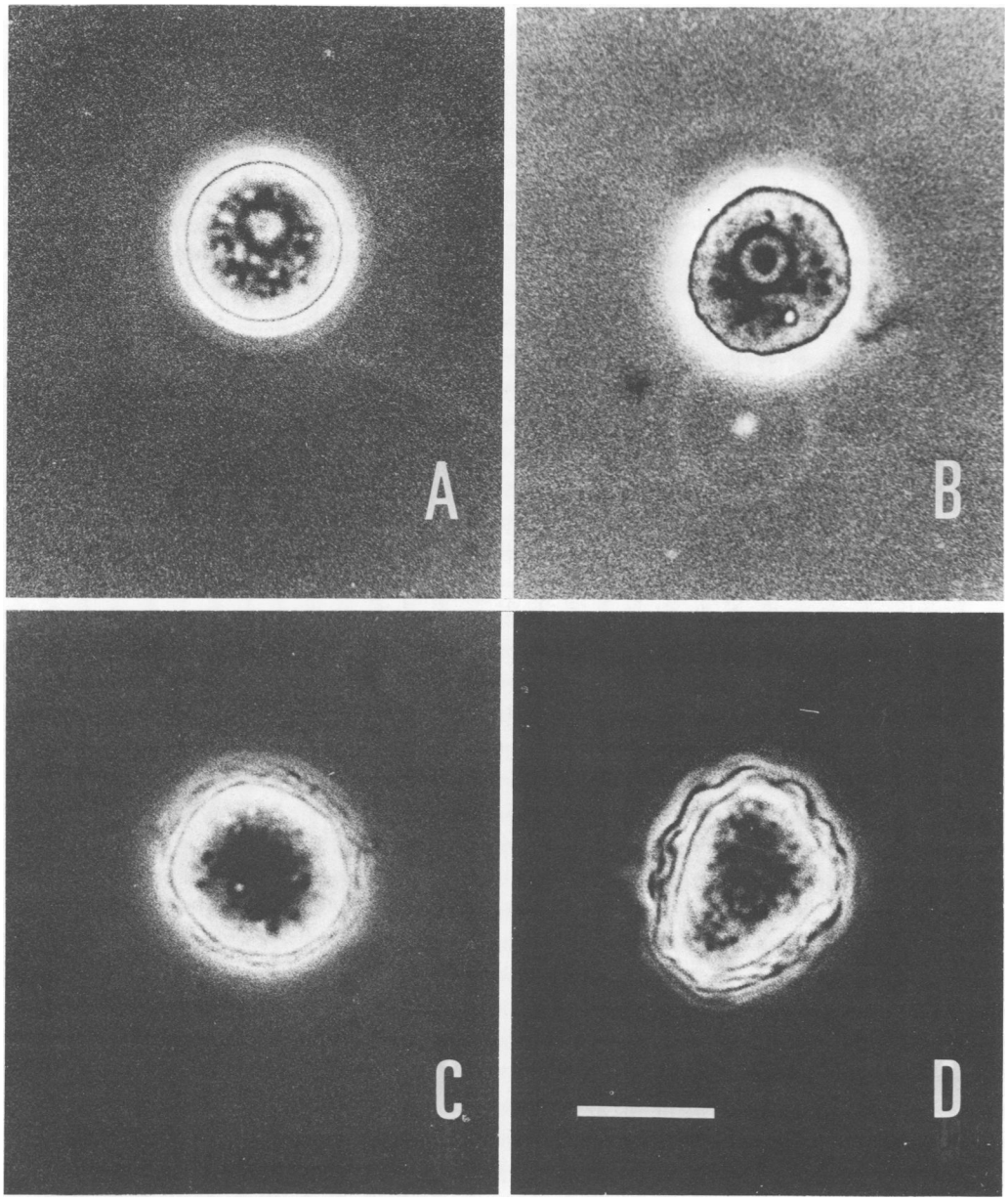


FIG. 1. *Phase-contrast microscopy of viable cysts of (A) N. fowleri HB-1, (B) N. gruberi 1518/1e, (C) A. culbertsoni A-1, and (D) A. polyphaga 4A. Bar. 10 μm.*

curred. The filter was inverted onto NNE for culturing the amoebae. The plate is sealed with Parafilm (American Can Co., Neenah, Wis.) and incubated at 37 C. The plates were examined daily for amoebic growth for a period of 2 weeks.

## RESULTS AND DISCUSSION

Table 1 shows at which time the cultures were first negative. The residual chlorine concentration at that time is indicated for each experiment. In repeated experiments, we observed that the destruction time depended on the initial chlorine concentration; the residual concentration did not alter the contact time necessary for destruction of the cysts. Within the genus *Naegleria,* it appeared that cysts of pathogenic *N. fowleri* were more sensitive to chlorine than those of the nonpathogenic *N. gruberi.* At a concentration of 0.5 μg of free chlorine per ml, *N. fowleri* cysts were destroyed

TABLE 1. *Contact time versus chlorine content for destruction of cysts*

| Strain | Chlorine concn (μg/ml) | | First negative (h) |
|---|---|---|---|
| | Initial | Residual[a] | |
| *N. fowleri* HB-1 | 0.2 | 0 | 24 |
| | 0.5 | 0.3 | 1 |
| | 1.0 | 0.8 | 1 |
| | 2.0 | 2.0 | 0.25 |
| *N. gruberi* 1518/1e | 0.2 | 0 | 24 |
| | 0.5 | 0.1 | 3 |
| | 1.0 | 0.6 | 1 |
| | 2.0 | 2.0 | 0.5 |
| *A. culbertsoni* A-1 | 2.25 | 1.0 | –[b] |
| | 4.0 | 1.75 | 24 |
| | 8.0 | 5.0 | 24 |
| | 16.0 | 9.0 | 24 |
| | 40.0 | 40.0 | 24 |
| *Acanthamoeba* sp. 4A | 2.0 | 1.0 | – |
| | 4.0 | 3.5 | – |
| | 8.0 | 6.0 | 24 |
| | 16.0 | 16.0 | 1 |

[a] Concentration at the time the culture is first negative or after 24 h, when there has been no killing effect.

[b] –, Still positive after 24 h of contact time.

after 1 h of contact time, whereas the first negative culture for *N. gruberi* at this concentration was obtained after 3 h of contact time. At a concentration of 2 μg/ml, these figures were, respectively, 15 and 30 min for *N. fowleri* and *N. gruberi*.

When one takes into account that we tested 1,000 cysts/ml, it is obvious that the chlorine concentrations needed to destroy the cysts of *Naegleria* spp. lie well within the range of practicable chlorine dosage. As we know, short superchlorination up to 10 μg/ml is in common use in water supply plants using surface waters in Belgium. Further, it must be said that under field conditions the cyst density of *Naegleria* spp. is unlikely to be higher than 1/ml in municipal supplies. However, it is desirable to destroy the amoebae in water supplies to avoid accidental nasal infection and infestation of swimming pools fed by tap water.

On the other hand, the genus *Acanthamoeba* calls for far more attention by its high resistance to free chlorine. The pathogenic *A. culbertsoni* A-1 is more resistant to chlorine than the avirulent *Acanthamoeba* strain isolated from tap water. Both strains are insensitive to the chlorine concentrations applicated in tap water and swimming pools; a free chlorine concentration of 4 μg/ml does not destroy the *Acanthamoeba* strains after 3 h of contact time.

These results are in accordance with our findings that many isolates from tap water belong to the genus *Acanthamoeba*, since they cannot be destroyed by the chlorine levels in drinking water. The fact that the pathogenic *A. culbertsoni* is far more resistant is very important, since the cultures are still positive after 3 h of contact with 40 μg of free chlorine per ml.

It was always thought that *Acanthamoeba* infections were very rare, and more concern has been given to *N. fowleri*, since there are now more than 75 cases of *Naegleria* primary amoebic meningoencephalitis recognized throughout the world. However, cases of *Acanthamoeba* infections seem to be recognized at this time in countries such as Venezuela (Willaert, personal communication) and Zambia (2). Also in the United States the case reported by C. Sotelo-Avila (12) is now believed to be caused by *Acanthamoeba* sp. (9; De Jonckheere, personal communication to A. J. Martinez).

We have also been able to study three pathogenic *Acanthamoeba* strains isolated by F. A. Comer (Instituut voor Hygiëne en Epidemiologie, Gent, Belgium) in Belgium from swimming pools. These strains all killed mice when inoculated intracerebrally. Further experiments revealed that one strain also killed mice when inoculated intranasally (unpublished data). One of these strains was isolated from water with a bromine concentration of 0.8 μg/ml.

Results with other halogens (our unpublished data) indicate that *A. culbertsoni* A-1 cysts are not destroyed by bromine (0.4 to 1.0 μg/ml), iodine (2.0 to 5.0 μg/ml), and iodophore (2.0 to 5.0 μg/ml) after 24 h of contact time in artificially made swimming pool water (urea [Merck], 1 μg/ml; albumin [Poviet], 10 μg/ml; phosphate buffer [Sörensen], pH 7.5, hardness, 342 μg/ml). On *N. fowleri* also, bromine had no effect after 24 h with the concentrations mentioned. Only iodine and iodophore had a good cysticidal effect at the concentrations used. This may be due to the fact that they are less reactive with the urea and albumin, especially the latter. The results with chlorine in this artificially made swimming pool water were in accordance with the experiments herein described for *Acanthamoeba* and *Naegleria*.

In conclusion, viable *Naegleria* cysts are not to be found in clean water where low concentrations of chlorine are constantly maintained. *Acanthamoeba* cysts can only be prevented in drinking water by prolonged superchlorination when preparing the water and if there is no further contamination possible in the distribution network, but this seems an impossible task. Moreover, *Acanthamoeba* cysts cannot be killed in swimming water at the concentration allowed in swimming pools when cysts are in-

troduced by human carriers or by air. The isolations of F. A. Comer from swimming water and our isolations from drinking water support these conclusions.

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